OBSERVATIONS ON OSTEOGENESIS

WITH

SPECIAL REFERENCE TO ENDOCHONDRAL OSSIFICATION.

By

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OBSERVATIONS ON OSTEOGENESIS, WITH SPECIAL REFERENCE TO ENDOCHONDRAL OSSIFICATION.

In histogenesis few processes have been the subject of so much controversy as that of bone deposition and growth. It is with considerable diffidence that so difficult a subject is approached, but many current opinions are in need of reconsideration or even of readjustment, and if only because of this, there is justification in hazarding a few observations. Keith (Menders of the Maimed. Oxford Medical Publications) has sketched the history of this controversy, beginning a century and a half ago with Duhamel and Haller. The former first demonstrated that long bones increased in length by continuous increments at their extremities. He made no mention of the diaphyseal plates of cartilage. (MacEwen has recently introduced this term in place of the previous less accurate word epiphyseal. The

activity of this carticlage is related to the diaphysis and not to the epiphysis.) Dubreuil (Croissance au niveau du cartilage de conjugaison. Paris, C.R. Soc. Biol., 1913) confirmed Duhamel's observations, showing that there was no interstitial growth in the shaft of a long bone during its increase in length. Lead pellets were used in place of the ivory stylets of Duhamel. Radiography was employed by Dubreuil in the measurement of the intervals between the pellets. This observation of Duhamel (although he himself failed to mention the diaphyseal cartilage) led subsequent observers to a consideration of cartilage and its rôle in bone growth. Bones were known to be laid down in cartilage. This cartilage disappeared. There were two possibilities - either cartilage was forming bone, or bone was being formed at the expense of cartilage. The majority of investigators came to the latter conclusion, an hypothesis startling if only from the point of view of a physiological error in economy. That cartilage plays a part in growth in length has

never been disputed, but the question of growth in thickness is still a subject of much discussion and investigation.

Duhamel originally attributed to the periosteum osteogenic function, and to it ascribed circumferential increase in long bones. Syme and Ollier produced experimental evidence in favour of this hypothesis (Keith: Menders of the Maimed). MacEwen has subsequently produced stronger experimental evidence to disprove it, or more accurately perhaps, has produced evidence to show that bone grows in the absence of periosteum. MacEwen's work has been confirmed by Gallie and Robertson (Brit. Journ. Surgery, VII, 1920, 211). Syme and Ollier may conceivably have removed bone tissue with their periosteal grafts. There was no possibility of MacEwen including periosteum with his bone grafts. He concludes that periosteum plays no part in the production and regeneration of bone, and that it functions merely as a limiting membrane

(MacEwen: Growth of Bone). Goodsir,(Anatomical Memoirs of John Goodsir. By William Turner. II.1868. 470) from a pathological standpoint, in the consideration of the shell of new bone surrounding a necrosed shaft resulting from osteomyelitis, came to the conclusion that "periosteum does not possess an independent power of forming osseous tissue." The evidence of MacEwen in his experimental treatise, "The Growth of Bone" - an inquiry into the development and reproduction of diaphyseal bone, together with three or four decades of experimental surgery - constitutes a most valuable record of observations on osteogenesis. He has demonstrated conclusively that bone may live without its periosteum, and further that bone as a separate tissue is capable of extensive proliferation. He regards interstitial growth as a potentiality of bone tissue, for example, when a pin is driven into bone and removed, the resultant gap is filled by the proliferation of the surrounding osseous tissue. Circumferential thickening he describes as a manifestation

of interstitial growth. Much evidence has been produced in support of MacEwen's contention that periosteum is merely a limiting membrane. Keith (Menders of the Maimed) associates Goodsir with the initial use of the term limiting membrane. Morley (Brit. Journ. Surgery, VII, 1920, 178) has produced experimental traumatic myositis ossificans by removing areas of periosteum and crushing the neighbouring muscles. He effected a cure by the grafting of autogenous fascia. MacEwen has further observed that in fractures where the periosteum is torn more callous is thrown out than in cases of fracture where the membrane is intact. Keith and Hall (Brit. Journ. Surgery, VII, 1920), in a study of the infection and repair of bones in the War Office Collection resulting from the recent European embroilment, have found bone masses devoid of periosteum forming new bone.

Histological evidence discloses osteoblasts in the subperiosteal space. MacEwen, and Gallie and Robertson contend that these osteoblasts come from the

Haversian canals. The other obvious possibility is that they are formed by the periosteum. Any problem of tissue formation is essentially a cytological problem. Experimental evidence leads mainly to an appreciation of function. Appreciation of structure is dependent of a study of the cytomorphosis of the elements from which a tissue is derived and a study of its evolution. Osteogenis is intimately associated with the evolution of the osteoblast. In endochondral ossification the usually accepted hypothesis is, that the osteoblast, along with bloodvessels and in company with the osteoclast, effects the disintegration of the cartilage, replacing it with bone. Geddes (Journ. Anat. and Physiol. XLVII, 1913) concludes that the osteoblast is derived from the ectoderm, having migrated through the periosteum into the cartilage cells. Todd (Journ. Anat. and Physiol. XLVII, 1913) regards the osteoblast as a fibroblast or connective tissue cell, which has undergone characteristic changes and may or may not have passed through the chondroblast

stage. MacEwen in a recent work (Growth and Shedding of the Antler of the Deer: Maclehose, Jackson and Co. Glasgow, 1920) has shown that osteoblasts are formed either directly from a connective tissue syncytium or through an intermediate stage of cartilage; from which he infers they emerge as naked nuclei. He thus regards the cartilage corpuscle as an osteoblast awaiting release and the arrival of the blood stream in order to obtain ossein for redisposition. The process in endochondral ossification is not parallel to the simple process described by MacEwen in deer antlers. The most striking cytological activity is observed in the cartilage cells. Retterer (Evolution du cartilage transitoire. Journal de l'anatomie et de la physiologie, 1900, p.467) has described this activity and the resultant cytomorphosis. Working independently without knowledge of this work, my conclusions are practically identical with his. With the hope of confirmation and extension rather than with the spirit of advancing a new point of view this

thesis is submitted in thebelief that where opinions are so divergent accumulated evidence alone leads to unanimity.

EVOLUTION OF THE CARTILAGE CELL.

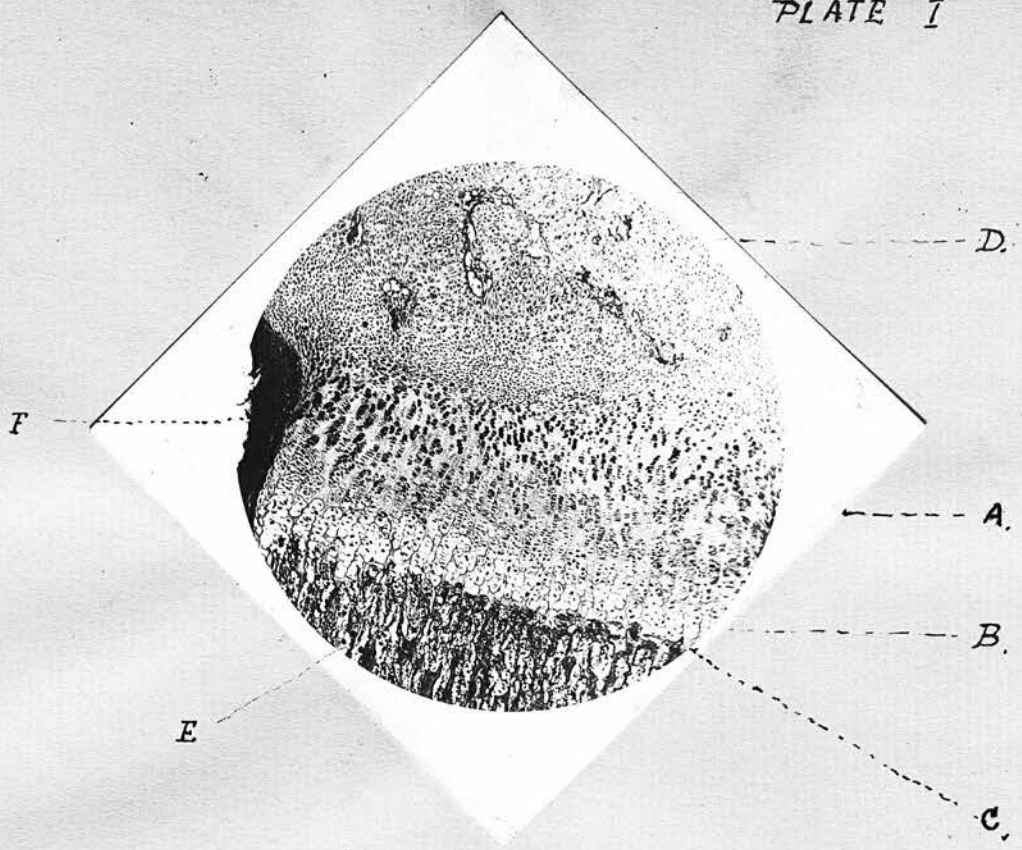
In the embryonic state thetchanges in the primitive connective tissue of the chondrogenetic areas are in the main extracellular changes. The syncytium originally resembles Wharton's jelly. In the meshes of the network is a foamy protoplasm, and in the nodes of the network are embedded the connective tissue energids. Four main changes are observed (Geddes. Journ. Anat. and Physiol., XLVII, 1913):

1. The fibrils of the network become less evident.
2. The connective tissue energids increase in number.
3. The energids group themselves at regular intervals and appear as naked nuclei, or as cells with a very small amount of cytoplasm.

4. Chondromucin appears in the exoplasm as a secretion of the cell to form a capsule.

Collectively these capsules form the cartilage matrix. The tissue which ultimately forms the periosteum, through the stage of perichondrium, is indistinguishable from the connective tissue syncytium of the chondrogenetic area and the adjacent perimysium. The differentiation from the cartilage area is one of gradation, the connective tissue cells of the perichondrium merging into the cartilage area; the transition being marked by a gradual increase in the amount of chondromucin in the exoplasm. This is the stage of perichondrium. Geddes describes a definite space, but I have not observed this until a later atage, when the cartilage is further deffenentiating into bone and the true periosteum is delimited.

PLATE I



Femur of Rabbit (foetal) decalcified.

A Serial zone.

B. Hypertrophic zone.

C. Hyperplastic zone.

E Bony Trabeculae. shewing continuity with cartilage trabeculae

F. Perichondrium. No subperichondral space is evident. Cells of the serial and hypertrophic zones are immediately subjacent.

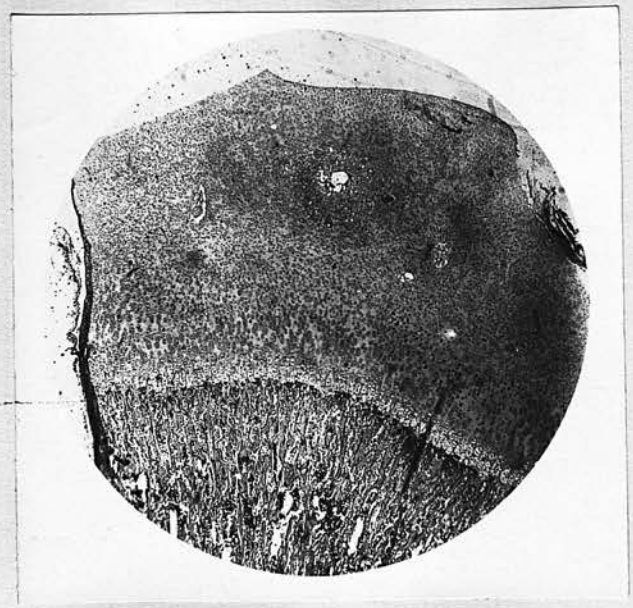
D. Epiphysis; shewing blood spaces and cartilage cells in transition prior to commencing ossification

CYTO-MORPHOSIS OF THE CARTILAGE CELL IN ENDOCHONDRAL OSSIFICATION IN THE DIAPHYSIS.

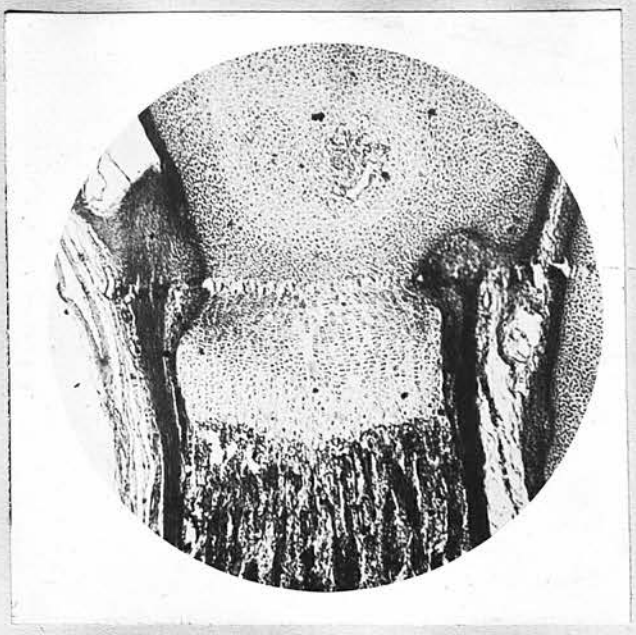
The first change observed is the multiplication by mitosis of the resting cartilage cells, whereby they enlarge slightly and separate, each new cell becoming completely invested with chondromucin after the process of separation.

The succeeding phase in cellular activity is marked by an arrangement in rows, parallel to the long axis of the diaphysis. In rapidly fixed specimens secured immediately after killing (vide Technique) mitotic figures are numerous. In the formation of these rows longitudinal trabeculae of chondromucin are formed. Filamentous transverse strands of chondromucin appear between each separated cell. This layer of cartilage cells is termed by Retterer the serial zone. In applying descriptive terms to the changing areas of cartilage cells those adopted by Retterer are used. The cells of this serial zone are characterised by their flattened appearance, lying transversely to the

PLATE ii.



Foetal Rabbit. (Tibia). decalcified
Shewing the continuity between
perichondrium and periosteum. (at A)
The gradation between the resting
cartilage and the transition zones is
well seen.

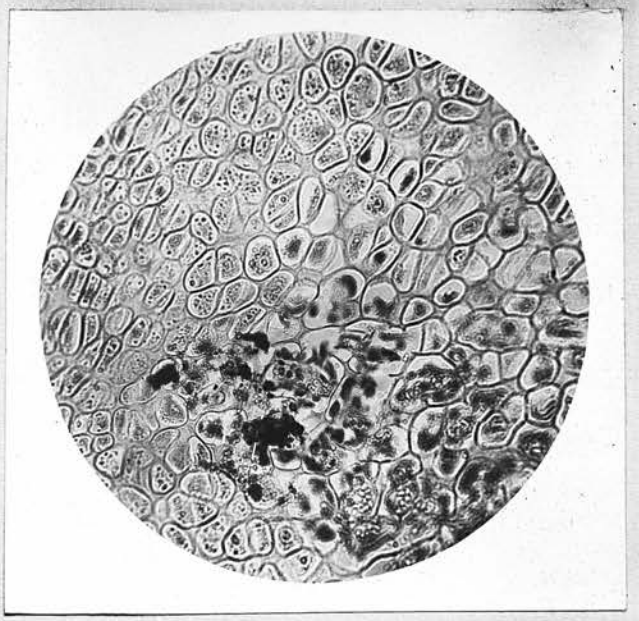


Foetal guinea pig (distal extremity of Radius).
not decalcified (Mallorys stain).
Ossification is proceeding in the epiphyses.
the cartilage shewing an external
hypertrophic zone. (X artifact produced by
the razor). The cells of the hypertropic zone
in the diaphysis are well preserved;
compare with plate I where the
hypertrophic zone is represented
mainly by empty cell spaces, and
occassional cell debris.

long axis of the diaphysis. The mitotic figures also occur in this transverse axis. The nuclei show a great affinity for acid fuchsine.

CELLS OF THE HYPERTROPHIC ZONE.

These cells are sometimes supposed to undergo disintegration, or ingestion by osteoclasts. The stellate and shrunken appearance so often observed is due to changes during fixation (vide Technique), or post mortem degeneration. Even after adequate fixing, rapid hardening and dehydration will cause the cell membrane to retract from the surrounding matrix. It is because of this shrinkage that the cell frequently appears as a mass of débris or even disengages completely from its space. The nucleus is large, 9-12μ, and stains well with acid fuchsine and iron alum haematoxylin. The nucleoplasm is granular, and masses of it appear to aggregate at the periphery of the nuclear membrane. The cytoplasm is reticular, with



Foetal (guinea pig) Oblique section through the three transition zones.
The granular and vacuolated cytoplasm of the cells of hypertrophic zone is well seen. Only occasional nuclei are evident in these cells. Owing to their large size the nucleus are only occasionally cut through in section.
Several are observed erupting into the hyperplastic zone. The large dark masses in the lower left quadrant are accidental debris accumulated during the photographic process.

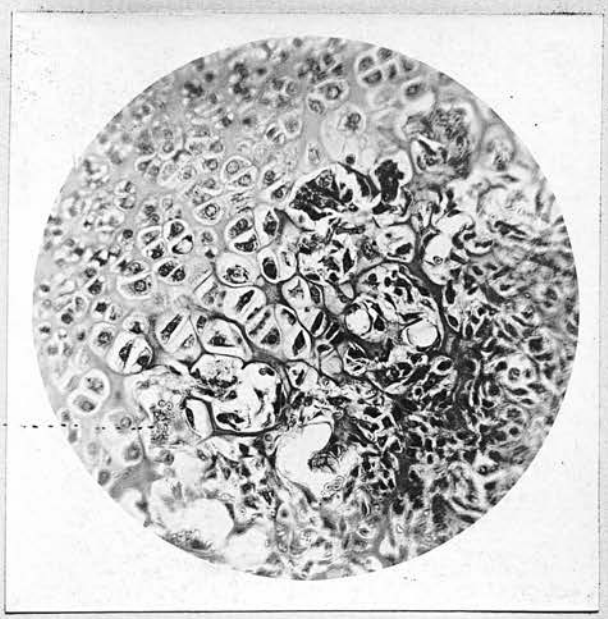
PLATE V.

Foetal guinea pig carpal bone (Mallory's stain) same as plate iv (magnified x600). Syncytial masses, branched cells and a clump of erythrocytes are seen emerging from a granular mass (A).

occasional granules, and globules of protoplasm with an affinity for orange G. Vacuolation is evident in the meshes of the reticular protoplasm. The transition from the serial zone is effected by an increase in the size of the cells. The longitudinal trabeculae of cartilage matrix separate the rows of cells, forming a well defined barrier. The transverse trabeculae which were undergoing successive readjustments in the serial zone form straight strands more or less obliquely placed. As the cells of the hypertrophic zone increase in size these latter strands become more and more attenuated. Rupture finally occurs and the cell is liberated between the longitudinal trabeculae.

HYPERPLASTIC ZONE.

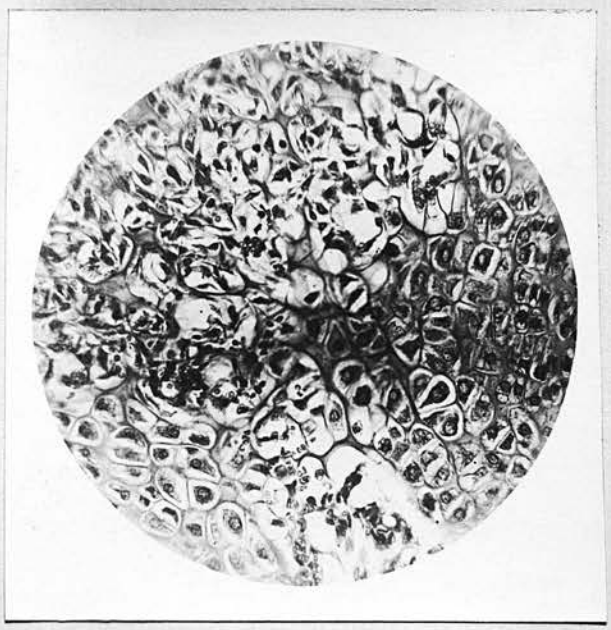
The liberation from the cartilage space is followed by a rapid metamorphosis, resulting in a hyperplasia of the cell mass. The cytoplasm forms a branching syncytium, in which the granules observed



Foetal guinea pig (Distal epiphyses of radius) (Iron-alum-haematoxylin counter stained with Van-Gieson's stain). Transition between hypertrophic and hyperplastic zone.

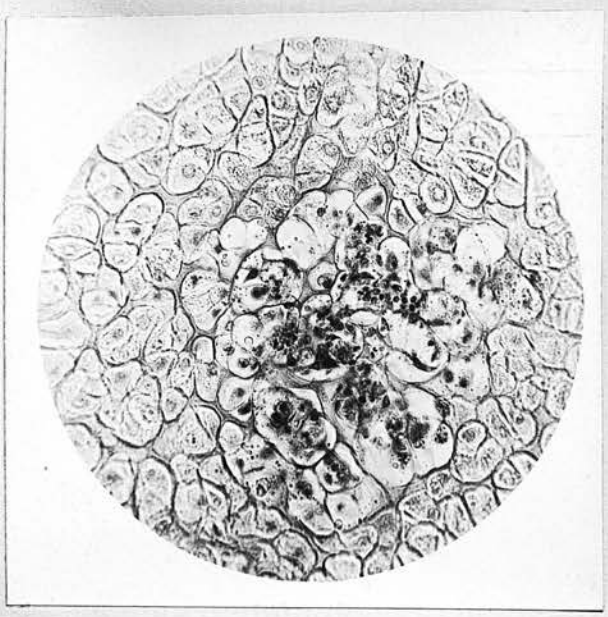
A. erythrocytes forming in a granular mass.

Same as plate VI but higher magnification. Cells of hypertrophic layer erupting. A liberated nucleus is observed in the middle of the plate. Branched vasoformative cells are making their appearance in the syncytium.

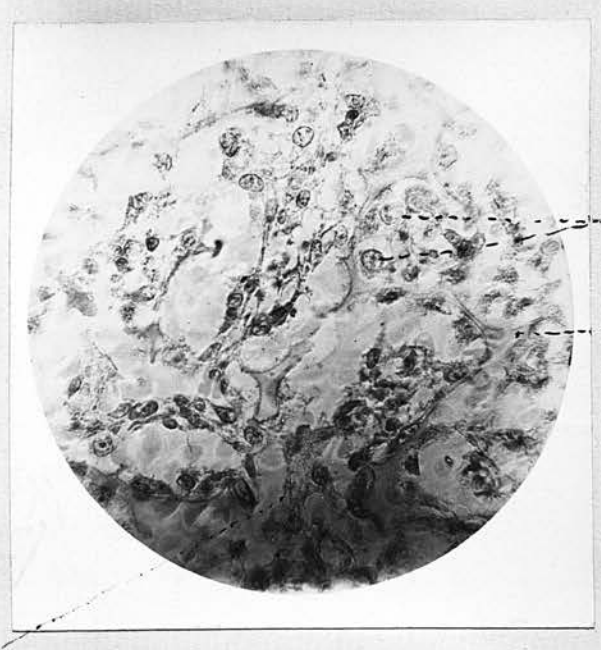


Foetal guinea pig. (same section as plates VI and VII.) Illustrating the transition stage at the line of ossification. The cytoplasm of the hypertrophic cells is not so well fixed as in previous plates.

PLATE IX.



Foetal guinea pig. (Mallory's stain).
Showing the appearance of the
syncytial masses differentiating into
cellular elements.



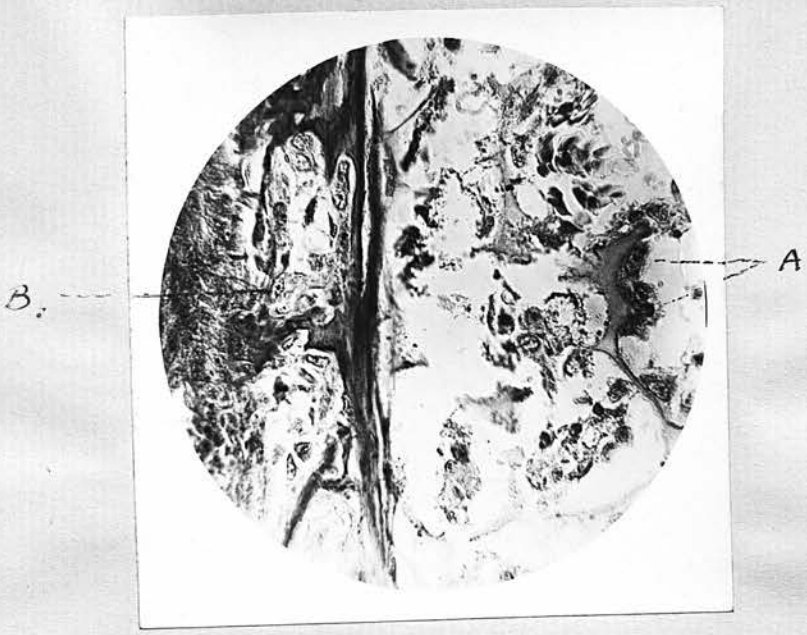
Femur. (foetal Rabbit). Mallory's stain.
Transverse section through the hyperplastic layer.

A. Cartilage trabeculae.

B. Osteoblasts migrating from the syncytial masses to the cartilage trabeculae.

C. Cells emerging from a syncytial mass.

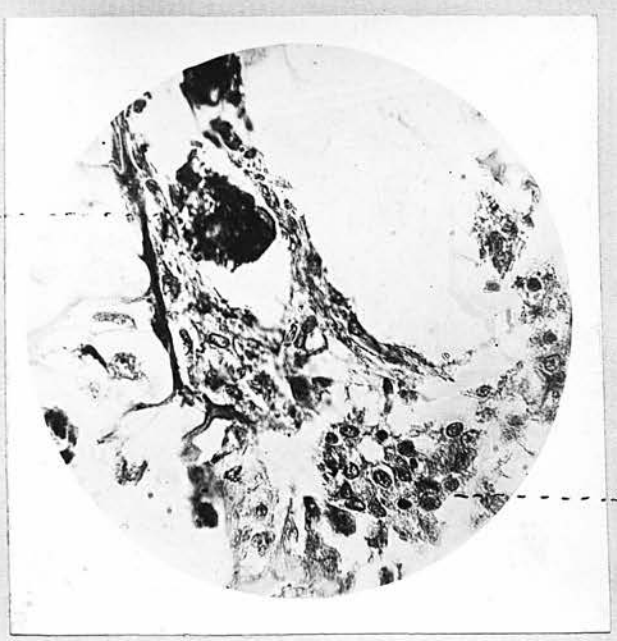
PLATE. XI



Foetal rabbit. Transverse section through the hyperplastic layer. in series with previous plate

A osteoblasts depositing osseni on cartilage trabeculae.

B. Migration of osteoblasts into the subperiosteal space.



Transverse section through Hyphoplastic layer of the femur of a foetal rabbit.
A. Cells differentiating from a syncytial mass.
B. A granular mass prior to differentiation.

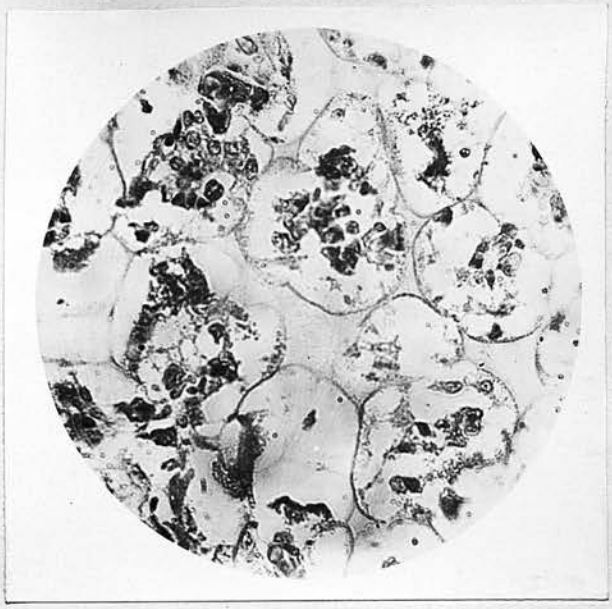
in the hypertrophic zone aggregate into globules. These granules appear to be secreted by the nucleus of the changing cartilage cell, and stain very densely with acid fuchsine. The globules formed by the aggregation of the granules finally appear as fully formed erythrocytes. No leucocytes are formed at this stage, nor are they found in the hyperplastic zone. They evolve subsequently from the mesenchyme cells which are described below. Schafer (Essentials of Histology, 1916) has described the formation of erythrocytes from granular masses similar to the process described above. The nucleus separated from its cytoplasm appears to undergo a definite lobulation and small ovoid masses are segregated: these enlarge and form a mesenchyme syncytium consisting of mesoderm cells varying in size according to the stage of differentiation. From this mesenchyme syncytium arise the osteoblasts and branching vasoformative cells, and later the blood elements of the bone

marrow. The fully formed erythrocytes appear finally in clumps, the branched cells of the syncytium migrate around these clumps, and the typical appearance of a minute capillary results. The osteoblasts when fully formed migrate to the walls of the longitudinal trabeculae, availing themselves of an existing scaffolding on which to build.

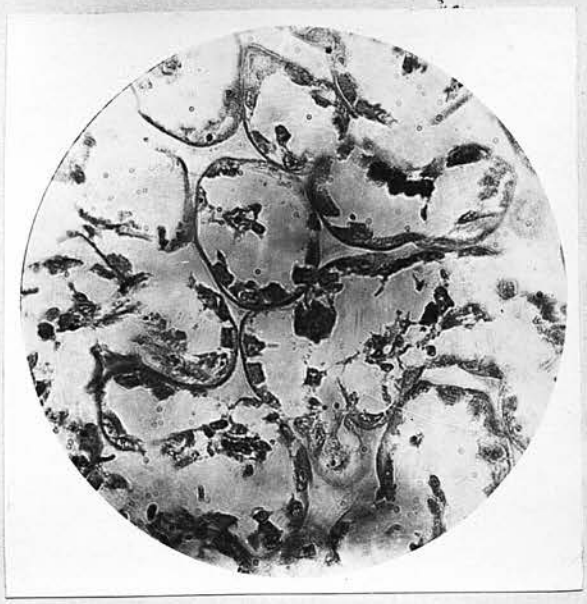
ENDOCHONDRAL OSSIFICATION IN THE EPIPHYSES.

Most epiphyses of long bones are atavistic epiphyses. Morphologically and physiologically considered they are independent of the diaphyses. Here the periosteum can have little claim to the production of the osseous tissue, as the original deposition of bone is far removed from this structure. The irregular bones of the carpus and tarsus show the same central deposition. The changes in the cartilage cells are similar in sequence to those observed in the disphysis except that the arrangement of the zones is centrifugal. Growth occurs by expansion, by a

Foetal Rabbit. Transverse section through the hyperplastic zone (Femur.).
A Granular masses after the eruption of the cartilage cell of the hypertrophic layer. (Compare the two following plates).



Oblique transverse section through the hyperplastic zone of the femur of a foetal rabbit (Same section as previous plate but at a slightly lower level). Erythrocytes are seen appearing in the granular masses. The mesenchyme cells are also well seen.



The same section as the previous two plates. but at a different level. The osteoblasts have differentiated from the mesenchyme syncytium and migrated to the cartilaginous trabeculae.

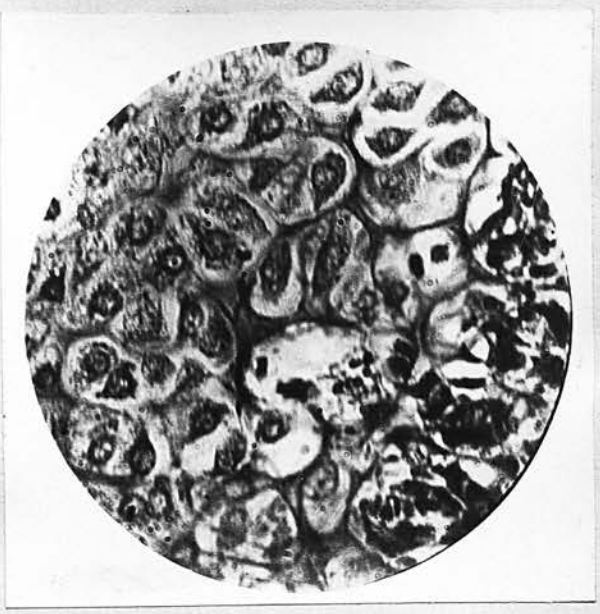
multiplication of cartilage cells at the periphery, and an increase of the transverse diameter of the diaphyseal plate. Before ossification begins other modifications occur in the cartilage of the epiphysis, whereby a solution of the chondromucin is effected and spaces are formed into which the naked cell erupts. The cytoplasm becomes granular, and a syncytium is formed consisting of vasoformative branched cells. Erythrocytes are formed by aggregation of granules and extruded. The branched cells unite to form capillaries. Two or three capillaries are often formed in one such space. These spaces are as a rule irregular in outline and on serial section appear to end blindly. Osteoblasts do not make their appearance in this cytomorphosis. These spaces were never observed to communicate with the perichondral tissues prior to ossification. In the distal epiphysis of a radius of a foetal guineapig however, a definite channel was traced in serial sections from the central

Vertebra (Guinea pig Mallory's stain x600)
Stage of eruption. The dark areas are masses of granules which have stained intensely with acid fuchsine.

of ossification to the perichondral space. No bloodvessels existed in this space but osteoblasts were seen lying freely. A few were observed apparently emerging into the perichondrium at its junction with the cartilage. There was no evidence of their production in the perichondrium. This observation gave rise to a thorough investigation of the subperiosteal osteoblast.

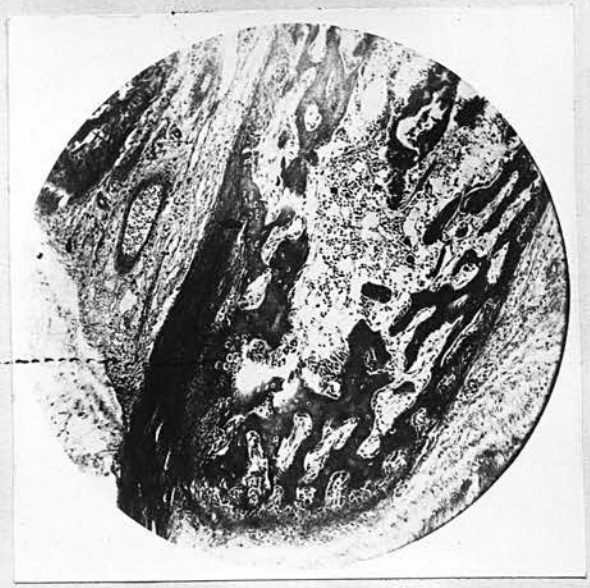
THE ORIGIN OF THE SUBPERIOSTEAL OSTEOBLAST.

There is no definite histological evidence to show that periosteum does not form osteoblasts: on the other hand there is less histological evidence to show that it does do so. The presence of osteoblasts in the subperichondral space is undeniable. Their presence in the subperiosteal space is still less refutable. I have already pointed out that on one occasion a channel was observed in the distal epiphysis of a foetal radius (guineapig) connecting the subperichondral area with the hyperplastic zone in an



Foetal guinea pig Radius Mallory's stain.
Stage of eruption shewing
transection from the hypertrophic
zone to the hyperplastic zone.

area undergoing ossification, and that osteoblasts appeared to be migrating from the hyperplastic zone to the subperichondral space. The migration in the opposite direction might equally well be argued were it not for the fact that only a few have reached the perichondrium, and that there is no indication of any cellular activity in that region to suggest the perichondrium as the site of their formation; whereas the hyperplastic area illustrates the whole process of osteoblastic transition exceedingly clearly. This evidence though slender suggests a way in which osteoblasts may gain the subperichondral space of the growing epiphysis. There is more positive evidence for considering the origin of the osteoblasts in the subperiosteal space. The circumference of the hyperplastic zone is surrounded with dense cartilage matrix in which are seen cartilage cells undergoing the cytomorphosis previously described. These cells in question usually erupt from their cartilage spaces



Metacarpal Foetal gumia pig Mallorys stain
A. A nest of cartilage cells
surrounded by a deposit of osseni,
erupting into the intertrabecular areas.

nearer the centre of the diaphysis than the level of the hyperplastic zone. Indeed some are delayed in their eruption until they are situated in the centre of bony trabeculae deep in the ossified area of the diaphysis. The majority erupt from the cartilage matrix at the junction of the perichondrium with the periosteum. Perichondrium only becomes periosteum as ossification proceeds from the centre of the diaphysis to the extremities. It is essentially a change in name corresponding to the movement of the hyperplastic zone as the formation of bone elements is effected. Osteoblasts would consequently be discharged into the whole length of the subperiosteal space as growth proceeds, and this process would continue until the disappearance of the diaphyseal plate. The subperiosteal space has frequent communications with the intertrabecular areas which ultimately become the Haversian canals. These communications are effected by the increase in diameter of the epiphyseal

PLATE XIX.

(Rabbit foetal Tibia Haematoxylin and Eosin)

A Periosteum

B Trabeculae of bone.

Osteoblasts are observed in process of migration from the intertrabecular areas into the subperiosteal space

The subperiosteal space is well marked. (C)

plate as growth occurs, resulting in the formation of cartilaginous trabeculae which overlap circumferentially. Through these gaps osteoblasts can be observed in stages of migration from the intertrabecular areas into the subperiosteal space. This contention is liable to the opposite interpretation were it not for the fact that the intertrabecular areas are seen to be crowded with cells, and the periosteum evinces no corresponding sign of cellular proliferation.

THE OSTEOCLAST.

I have found no cellular elements either of the structure or function asfribed to this cell. In no section observed by me has it made its appearance in the hypertrophic zone. Occasionally masses of osteoblasts become fused by ossein and ressemble a multinuclear mass, but examination under high power renders the precise explanation.

Todd (Journ. Anat. and Physiol., XLVII, 1913)

considers osteoclasts to be masses of pre-osseous tissue, artificially separated from the fully ossified bone during its preparation. It is possible that the mesenchyme syncytium resulting from the nuclear change of the cartilage cell might have been confused with a multinucleated cell.

TECHNIQUE.

These observations were carried out on human foetal bones varying from four and a half months to full time. Rabbits, foetal and adult, and foetal guineapigs were also used.

Fixation of Tissue. The main difficulty was to fix the large cells of the hypertrophic zone. Ordinary fixatives and procedure failed. The best results were obtained by killing the pregnant animal, exposing the uterus immediately, and delivering the foetus in caul. The tissues required were rapidly dissected with a minimum of handling (removal of the skin was essential) and placed in Zenkers fluid heated

to 37°C. to which 5 cc. of glacial acetic acid had been added to every 100 cc. of Zenkers, and well mixed, immediately before immersing the tissue. The specimen bottle was tightly corked to prevent evaporation of the glacial acetic acid. Forty-eight hours was found to be the optimum time for fixation.

Decalcification. Perenny's solution was used, but failed to give satisfactory results owing to the shrinkage of the cellular elements. Decalcification was ultimately discarded.

Hardening and Embedding. Graduated alcohols were employed in hardening and dehydration. The tissues were cut into small pieces to minimise the time in the alcohols. Benzol was used in preference to chloroform in clearing. Two to four hours was found to be the optimum limit. The tissues were embedded and cut in paraffin with a melting point of 54°C.

Staining. The stains used were:

i. Haematoxylin and alcoholic eosin.

ii. Mallory's connective tissue stain in three stages.

iii. Iron-alum-haematoxylin counterstained with Van Geison.

iv. Leishman's stain.

The best differentiation was obtained with Mallory's stain.

CONCLUSIONS.

1. In endochondral ossification the osteoblast is evolved by the cytomorphosis of the cartilage cell.

2. Growth in length of a long bone is effected by the activity of the cartilage cell in producing osteoblasts, and in moulding cartilaginous trabeculae on which the osteoblast secretes its ossein.

3. Erythrocytes are formed from granular masses deposited by the cartilage cell during its cytomorphosis.

4. The blood spaces of the epiphysis are formed by the cartilage cells undergoing characteristic modifications.

5. Subperiosteal osteoblasts migrate from the chondral and osseous areas into the subperiosteal space.

6. Osteoclasts as differentiated cellular structures have not been observed to play any part in osteogenesis.

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